TECHNIQUE FOR THE EXAMINATION OF SEEDS FOR THE PRESENCE OF SEED-BORNE FUNGI

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(With Plates I–III)

That seeds of agricultural and horticultural crops can carry fungi in the form of both mycelium and spores which may be parasitic and produce serious crop losses through causing disease is only too well known. To the category of the cereal smuts which may be taken as the classical example must now be added species of *Helminthosporium*, *Fusarium*, *Colletotrichum*, *Botrytis*, *Phoma*, *Septoria*, *Phialea* and many other fungi, while to the cereal hosts can be added the large majority of seeds of those species and varieties of plants used for cropping purposes. In many countries lists have been prepared of the seed-borne fungi with particular reference to those which can behave as parasites. In the Netherlands, Dr L. C. Doyer has made a special study of the problem as it is related to seed testing and has published her observations in her *Manual for the Determination of Seed-borne Diseases* (Doyer, 1938).

In Northern Ireland an attempt has been made, not so much to list those fungi which can and do behave as seed-borne parasites, but to devise rapid and accurate technique whereby seeds can be readily examined and their potential state of health determined in so far as it may be related to the presence of seed-borne parasitic fungi. It was decided that such technique should aim at providing results of both qualitative and quantitative value. The underlying reason for undertaking the work was as follows. Should it ever be considered that in the interests of crop hygiene some knowledge of the potential state of the health of seeds should be available in addition to data concerning their purity and germination capacity, then suitable technique to allow of this being done would be necessary. The methods employed would take their place as laboratory routine and would have to be capable of being operated by trained laboratory assistants. Experience has shown that the elaboration of such technique requires some research and patience, and that fresh difficulties are liable to arise whenever a new problem is approached. Nevertheless, it has also shown that these problems are eventually capable of solution and there appears to be no good reason why, in the course of time, satisfactory technique will not be built up which will allow of the speedy and effective routine examination of all seeds for their state of health whenever this may be necessary. Such an examination, taken together with relevant information concerning the growing crop from which the seed is produced (when this is required), should go far towards raising the standard of seed quality and thereby bringing about crop improvement. In all the methods so far devised the aim has been not only to determine whether the parasite is present on the seed but also to ascertain whether or not it is present in a viable state.

For the purpose of this paper three particular examples have been selected and some account is given of the way in which the problems raised by each have been approached and dealt with in Northern Ireland. The examples are: *Helminthosporium* disease of oats; the seed-borne parasites of flax; and Blind Seed disease of ryegrass.

THE TECHNIQUE

Helminthosporium *disease of oats*

This disease is caused by *H. avenae* which is carried by the seed (usually on or in the glumes) as mycelium and sometimes as spores. The problem was to determine with accuracy all those seeds in a particular sample which carried the parasite. It has been shown (Muskett, 1937, 1938) that this may be done by sowing the seeds in soil under controlled conditions of temperature and moisture content and examining the seedlings for the presence of the disease, but this method was laborious and altogether unsuitable for the purpose required. Some other method of approach to the problem was necessary. An examination of the dry seeds is unsatisfactory, as spores of the fungus are seldom found and the resemblance of the mycelium of *Helminthosporium* to that of other dematioid species renders diagnosis by this means both dangerous and unreliable. It may be mentioned here that the colour of a seed sample does not form a reliable guide as to the extent of its contamination with *Helminthosporium*; some of the brightest samples of seed oats may be heavily contaminated, while in others which are dirty in appearance and of poor colour the fungus may be absent. Fortunately, it is possible to get the fungus to develop and sporulate on the seed, and it was decided to adopt the production of living spores by the fungus on the seed as the criterion for determining seed contamination. By plating out the seeds on damp filter-papers lining the bottoms of Petri dishes and incubating them for nine days at 22° C. the fungus was given the necessary conditions and time to sporulate, and it was found that, after cutting away the shoots, the seeds could be rapidly examined for the presence of spores using a wide field binocular microscope. Spore formation did not occur in every case of contamination and it sometimes did not take place freely, but by exposing the seeds for a short period to the light of a Hanovia quartz mercury vapour sun lamp in accordance with the suggestion of Dillon Weston (1933) it was found that spore formation was significantly increased. Even so, a few contaminated seeds may fail to show spore development, especially in freshly harvested samples. In seed samples stored over year in the laboratory the percentage of contaminated seeds has frequently been found to have increased after storage. Although this phenomenon has not yet been fully explained it would seem that after a period of rest under dry conditions, the mycelium of *Helminthosporium* attains a condition where it produces spores more readily when submitted to suitable conditions. In many seed-borne moulds the reverse is the case and a reduction in their incidence in a viable state is noticed with the passage of time in storage. In spite of this slight disadvantage, whereby the estimate of the percentage contamination of a seed sample with *Helminthosporium* may tend to err on the low side, the method has given excellent results in practice and has been found to be quite reliable for the purpose of seed examination. It can be carried out by a trained laboratory assistant and it takes only nine days to complete the test, a period which is well within the limits required by seed testing for purity of sample and germination capacity.

But the recognition of seed contamination by *H. avenae* and the oat smut fungi may not complete the story of the seed-borne parasitic fungi affecting this crop. In Northern Ireland, experiments dealing with seed disinfection have always purposely referred to the effects of disinfection in general terms rather than ascribing its value solely to the control of *Helminthosporium* and smut diseases. This was because beneficial results were frequently obtained where neither *Helminthosporium* nor the smut fungi were present on the seed. In so far as the oat crop is concerned the role of the seed-borne species of *Fusarium* has yet to be clearly elucidated, and when this has been done it may be found desirable to examine seed samples for contamination with these fungi. The method employed for *Helminthosporium* may not be suitable for this purpose, in which case the elaboration of further technique will be necessary either to deal with *Fusarium* spp. alone or, better still, to serve for all the seed-borne fungi affecting this crop.

Details of the method

First day. The sample of seeds is thoroughly mixed and the number required for examination purposes selected by the method of random sampling. If a high measure of accuracy is desired the test will require 500 seeds, but this number may be scaled down according to the purpose for which the test is being made. Petri dishes (9·0 cm.) are prepared by lining the bottom of each with two filter-papers which are moistened after being placed in position, by almost filling the dish with tap water and allowing the surplus water to drain off after the papers have become saturated. Sterilization of the dishes is usually quite unnecessary. The seeds are arranged (fifteen to each dish) with equidistant spacing in the dishes and the closed dishes are then placed in an open container, the mouth of which is afterwards closed with cloths saturated with water in order to prevent drying out. The container is placed in an incubator set to run at 22° C.

Fourth day. The dishes are removed from the incubator, the lids are taken off and the seeds irradiated for 20 min. A Hanovia sun lamp operating at a distance of 1 ft. (30·5 cm.) from the seeds is suitable for this purpose (Pl. I, fig. 1). According to Dillon Weston (1936) spore formation is induced by intense white visible light and not by the invisible ultra-violet rays. The dishes are then repacked in the container which is replaced in the incubator.

Ninth day. The seeds are examined for the presence of conidia of *Helminthosporium avenae*. The shoots are first removed with a sharp scalpel in order to facilitate examination. A most suitable microscope to use for this purpose is a wide field binocular model (paired eyepieces 15 ×, paired objectives 4 ×), giving a magnification of 60 × (Pl. I, fig. 2). Only one examination of the seeds is necessary. The glume apices are first examined, then the basal end of the grain and finally the remainder of the grain surface (Pl. I, fig. 3).

Seed-borne parasites of flax

The principle of the Ulster method for the examination of flax seed for its contamination with seed-borne parasitic fungi (Muskett & Malone, 1941) depends upon the fact that the parasitic fungi in question can be relied upon to grow out from the seed into a nutrient medium. The problem was approached in several other ways, some of which could be successfully operated and some of which failed, but none of the methods investigated could compare with the one described in simplicity and ease of operation, both of which factors are of the greatest importance if technique of this type is to be translated into large-scale routine practice. By using this method the presence of the following parasites on or in the seed may be readily detected—*Colletotrichum linicola* (Seedling Blight), *Polyspora lini* (Stem Break and Browning), *Botrytis cinerea* (Grey Mould), *Phoma* sp. (Foot-Rot) and *Fusarium lini* (Wilt). No medium has been found to be more suitable for the work than 2% malt extract agar, which has proved to be satisfactory for all the parasitic types investigated. Furthermore, the test only required five days, a period which is well within the limits needed for practical seed testing. In seed samples heavily contaminated with moulds the identification of the organisms may prove a little more difficult than is normally the case, but this has not proved to be a serious obstacle, and when once a competent laboratory assistant has been trained for the work a high degree of skill and rapidity of working may be attained. More than 20,000 samples of flax seed have been examined in Northern Ireland by this method during the past six years and there has thus been ample opportunity for testing it on a large scale. Microscopic examination of the colonies is seldom necessary; a glance at the obverse and reverse sides of the dish and a rapid inspection by transmitted light is all that is normally required. When it is said that *Pullularia pullulans* and *Polyspora lini* may be quite readily distinguished by inspection of the cultures, some idea of the general reliability of the method may be gained.

Details of the method

First day. The seed bulk is thoroughly mixed and a random sample containing many times the number of seeds required for the test is placed in a large watch-glass. Petri dishes (9·0 cm.) are prepared as for normal culture work and a thin layer of 2% malt extract agar (1·5% agar agar) is poured into each. When the agar has set, ten flax seeds are spaced equidistantly on its surface, the usual precautions being taken to prevent contamination of the medium with extraneous organisms. If strict accuracy is required, 500 seeds are used for each sample, but this number may be reduced according to the interpretation to be placed on the quantitative value of the result. Each seed is picked up with blunt-nosed forceps and dropped into position on the agar in the dish. The ends of the forceps are sterilized by dipping in methylated spirit and flaming after each ten seeds have been plated. This is because fungus spores are frequently present on flax seed, and it has been shown that there is a real danger of spreading contamination from seed to seed during the operation of plating unless frequent sterilization of the forceps is carried out (Pl. I, fig. 4). When plating has been completed the dishes are packed into an incubator set to run at 22° C. Great care and cleanliness must be continuously observed when this work is carried out constantly on a large scale over a prolonged period. Slovenly plating or the use of contaminated incubators may result in the necessity of repeating the examination of large batches of seed samples, particularly if contamination with such moulds as the *Monilia* stage of *Neurospora sitophila* or certain species of *Penicillium* become rife. For the decontamination and disinfection of incubators and small laboratories fumigation with formaldehyde, using a mixture of formalin (40%), and potassium permanganate has been found to be very effective.

Fifth day. The dishes are removed from the incubator and examined by eye inspection (Pl. II, fig. 5). Typical colonies of the five seed-borne parasitic fungi for which flax seed may be examined by this method are shown in Pl. III, figs. 9–13.

Blind Seed disease of ryegrass

The contamination and infection of perennial ryegrass seeds with *Phialea temulenta*, the organism responsible for Blind Seed disease, has assumed greater importance since the introduction of the more susceptible pedigree strains of this grass, and a simple method for the examination of seed samples for the presence of this parasite became necessary. Macrospores of the fungus are produced in abundance on the surface of the caryopsis of a contaminated seed and the observance of these spores forms a ready method for the examination of a seed sample. To make the examination each seed is placed in a drop of water on a clean glass microscope slide; one or both glumes are removed and the macrospores float out in large numbers into the surrounding water. Their presence is confirmed by a rapid microscopic examination of the slide using a magnification of 100 ×. Whereas this method has been found to be the most suitable and reliable for seed examination it cannot always be relied upon as an index of the viability of the fungus because the macrospores are comparatively short lived and usually lose their viability within six months of harvesting the crop. If, when the examination has been completed, the question of the viability of the fungus arises, information on this point can be gained by employing the method used by Neill and Hyde (1939) for the isolation of the fungus. The glumes are removed from the caryopses which are surface sterilized by immersion in 0·1 % mercuric chloride for ten minutes. They are then washed in sterile water, bisected longitudinally and the portions plated out on 2% malt extract agar. The fungus is slow growing but if it is viable, recognizable colonies showing copious macrospore production will be observed after incubation at 22° C. for seven to twelve days according to the age of the seed. The culture method is not suitable for the estimation of the percentage infection of ryegrass seed samples, because even when freshly harvested seed is used, where the fungus should be viable in every case, the estimate of infection will usually be found to be much lower than that given by microscopic examination. This may be explained in part by the killing of the fungus in some of the seeds by the process of surface sterilization and also by its non-exposure in some cases following upon the bisection of the seed. The microscopic method is therefore the most suitable for the routine examination of seed samples for percentage contamination, while evidence of the viability of the fungus may be obtained by the cultural method. Heavily infected seeds are normally opaque when examined by the diaphanoscope, and a suitably small sample for cultural work can be obtained by selecting these from the bulk with the aid of a diaphanoscope.

Details of the method

MICROSCOPIC METHOD (FOR PERCENTAGE CONTAMINATION). The necessary number of microscope slides (3 × 1 in.) are cleaned and three drops of water are equidistantly spaced on each. Using forceps, one ryegrass seed from the thoroughly mixed sample is placed in each drop. Five hundred seeds are used when a quantitatively correct result is required. One or both glumes are removed from the seed while in the drop of water by using forceps and a dissecting needle (Pl. II, fig. 6). After each seed has been dealt with the ends of the forceps and the point of the needle are carefully cleaned by rubbing with a duster. When this operation has been completed the slides are microscopically examined for the presence of macrospores, using a magnification of 100 × (Pl. II, fig. 7).

CULTURAL METHOD (FOR VIABILITY OF THE FUNGUS). As this method is qualitative rather than quantitative the number of seeds used is not of such importance and will depend upon the circumstances of the test. Heavily contaminated seeds are very suitable and may be selected by the use of a diaphanoscope if available. The glumes are removed and the caryopses are surface sterilized by immersion for ten minutes in a 0·1 % solution of mercuric chloride. They are then thoroughly washed in sterile water and bisected longitudinally, using a sharp scalpel which is sterilized at intervals as required and normally after dealing with each group of ten seeds. The portions are plated on 2% malt extract agar and are then incubated at 22° C. Colonies of *Phialea temulenta* will grow out from the infected seeds and can usually be recognized after seven to twelve days' incubation (Pl. II, fig. 8). As the fungus produces copious macrospores the observance of these serves as an aid to identification.

TECHNIQUE FOR THE EVALUATION OF SEED DISINFECTANTS

The technique here described was not evolved in the first place with the express purpose of using it to examine seeds for the presence of seed-borne parasites; it was evolved for use in a series of tests designed for the rapid and accurate evaluation of seed disinfectants. These tests form the laboratory side of the work entailed in assessing the value of seed disinfectants and they are operated on the following principle. It was assumed that if a method could be found for identifying the presence of a viable fungal parasite on a seed, and laboratory tests were made with this method using both non-disinfected and disinfected seeds, then a measure of the value of the disinfectant would be given by its effectiveness in suppressing the growth of the parasite being studied. This, indeed, proved to be the case and when the results obtained by the laboratory technique were compared with those obtained in the field over a three-year experimental period, a very high degree of correlation occurred between them. Thus it was found only necessary to experiment in the field with those disinfectants which show great promise as the result of laboratory testing and, even then, the reliability of the laboratory method allows the field work to be curtailed. This finding constitutes a very important economy both in time and expense, as the field side of such an investigation is a much more protracted and expensive undertaking. These results have been found to hold good in all the three examples under review, i.e. *Helminthosporium* disease of oats, the seed-borne parasites of flax and Blind Seed disease of ryegrass (Muskett, 1938; Muskett & Colhoun, 1942, 1943; Calvert & Muskett, 1945). By means of this laboratory technique a considered opinion upon the value of a seed disinfectant may be given within a period of a few days at any time of the year. Ineffective materials can be sorted out and discarded immediately and only those of the greatest promise need confirmatory testing in the field. The value of the application of this technique was clearly proved during the war when it was essential to try and find an effective seed disinfectant for flax. During the winter of 1939–40 it was found possible to investigate the behaviour of some hundreds of materials in the laboratory and as the result of this work a satisfactory disinfectant was found. Had field experimentation been the only method of approach to the problem, it would have taken a period of years to do the same work with only a proportion of the materials the values of which were assessed by this rapid technique.

Discussion

The work dealt with in this paper has been confined to the examination of true seeds for their contamination with seed-borne fungal parasites, but this forms only part of a much wider problem which is becoming of increasing significance in crop husbandry. Not only are seed-borne parasitic fungi responsible for crop losses; diseases caused by bacteria, deterioration by moulds, the carriage of viruses by the seed, damage by insects, deficiency diseases and physiological disturbances can all play their part by directly damaging the seed itself or by acting as the vehicle for the transmission of disease. There is also the grave danger of the transmission of parasitic organisms on detritus present as an impurity in a seed sample. Nor, with the rapidly increasing facilities for seed exchange, must the possible role of seed in spreading new diseases or making more widespread those which have become established, be overlooked. But crops are propagated by other means as well as by the use of true seeds, and these methods must also be taken into account. Tubers, bulbs, runners, rhizomes, roots and other organs of vegetative propagation may play the same role as the true seed and the ensurance of their good health has, in many instances, already received greater recognition than with seed. Broadly speaking, one of the major problems to be faced in the future of crop husbandry may well be the taking of more effective measures to ensure the health as well as the viability of all those plant parts which are used for the purpose of crop propagation. In order that an adequate examination for health may be made, suitable technique for the purpose must be evolved, and when this has been done and a wide range of technique easy of operation and relatively inexpensive to practise has become available, the approach to the problem will have been very much simplified.

The need for seed surveys from the standpoint of seed health is a real one, and it may be that the carrying out of such surveys will prove to be a necessary prelude to the routine examination of seed supplies. The health survey made for flax seed produced in the United Kingdom may be quoted as an example. The recent emergency allowed of this being done, and for the past three years some 3000–4000 samples of flax seed from all parts of the United Kingdom have been examined annually by the Ulster method for their contamination with seed-borne parasites. The results have not yet been published, but they are of no little interest. Briefly, they indicate that contamination with *Colletotrichum linicola*, *Polyspora lini* and *Phoma* sp. is much heavier in the area to the north and west of the Pennines than to the south and east. Unless a succession of very favourable seasons is encountered, the build-up of parasites increases with repeated sowings of seed from the same stocks. This fact in itself explains why it is that the climate in the north and west is unsuitable for flax seed production in so far as it encourages a build-up of seed-borne parasites which may constitute a limiting factor in successful seed production. *When it is stated that the purity of all these seed samples was of a very high order and that the germination capacity in every case was greater than* 90%, *the significance of seed health as a factor which is not determined by routine methods of seed testing becomes very apparent. In accordance with present seeds legislation every one of these samples would be acceptable as seed of the first quality.* Had it not been for the fact that a method of seed disinfection was discovered in Northern Ireland which prevented completely any likely crop damage by Seedling Blight (*Colletotrichum linicola*) and largely prevented damage by Stem Break and Browning (*Polyspora lini*), the United Kingdom flax crop during the period of the war would have produced less fibre to the value of £2,000,000. The cost of making such a survey must be considered as trifling when it is realized that the expenses would be more than adequately covered by the saving of a few acres of crop in the light of the knowledge so gained.

The general distribution in the United Kingdom of seed oats contaminated with *Helminthosporium avenae* has yet to be determined, and the same remains to be done for ryegrass contaminated with *Phialea temulenta*. Already, useful information is accruing from the examination of ryegrass seed samples on a large scale for the presence or absence of this parasite. But, national boundaries will not serve the cause of science and this work will not have been completed until it has been possible to make a thorough examination of seed stocks from crops grown over very wide territories—not, in fact, until such an examination has been made for every district and climate in which the crop is grown.

Finally, it is of interest to note that the technique here described was not deliberately sought with the object of seed examination for health and this in itself is a fact of no small significance. These methods were evolved during the process of endeavouring to discover rapid and accurate technique for the evaluation of seed disinfectants. Thus they assume a double importance because it has been shown in all the cases under review that, with but slight adaptation, they can be used to measure the value of a seed disinfectant as an instrument for the prevention of diseases likely to be caused by seed-borne parasites.

SUMMARY

1. Suitable technique for the examination of seeds for the presence of seed-borne fungal parasites is described. Such technique must be easily operated on a large scale, relatively inexpensive, and capable of being exercised by trained laboratory assistants. The three particular examples for which the methods employed are described in detail are: *Helminthosporium avenae* of oats, the seed-borne parasites of flax and *Phialea temulenta* of ryegrass.

2. The following points are discussed: (*a*) The significance of examining seeds and other plant organs used in crop propagation, for their state of health. (*b*) The value of making seed health surveys by employing the technique described. (*c*) The limitations of routine seed-testing methods for the determining of purity and germination capacity in providing an index of the health of seeds. (*d*) The use of closely allied technique for measuring the values of seed disinfectants.

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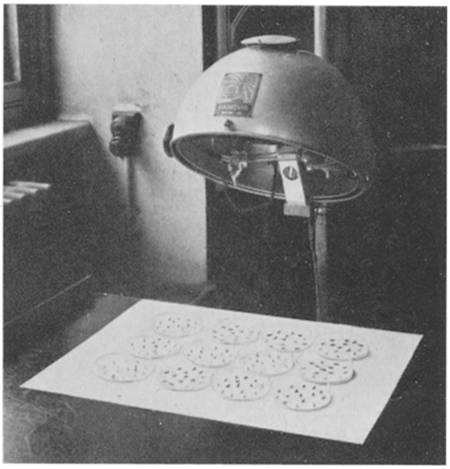

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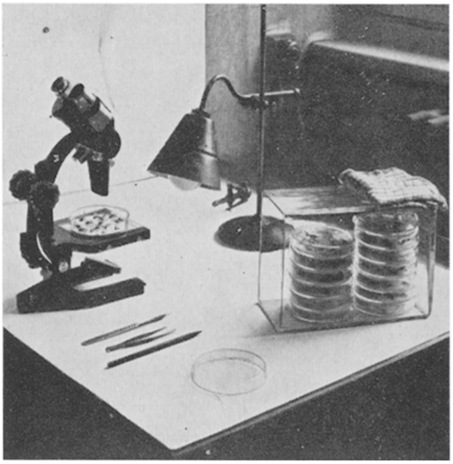

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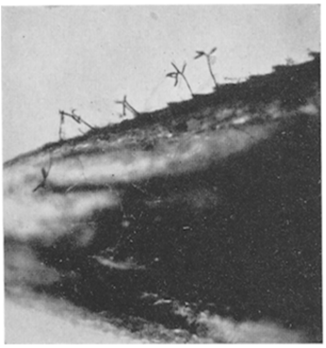

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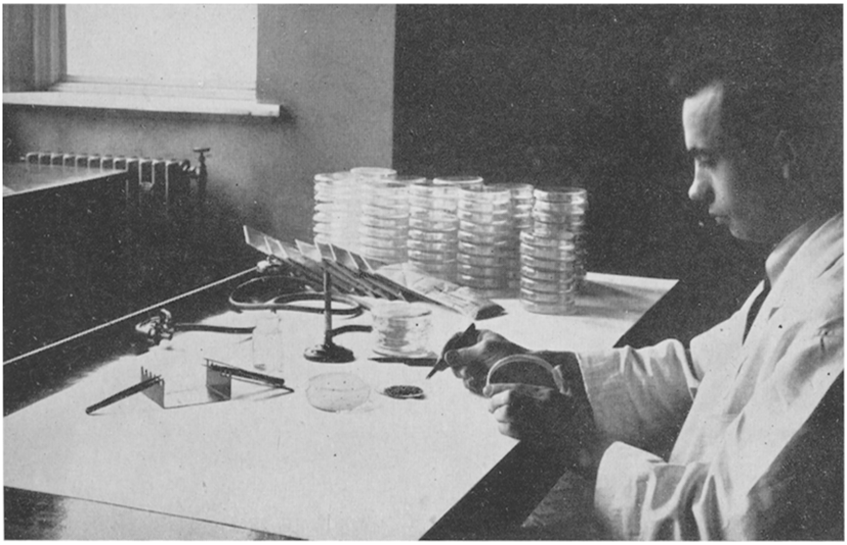

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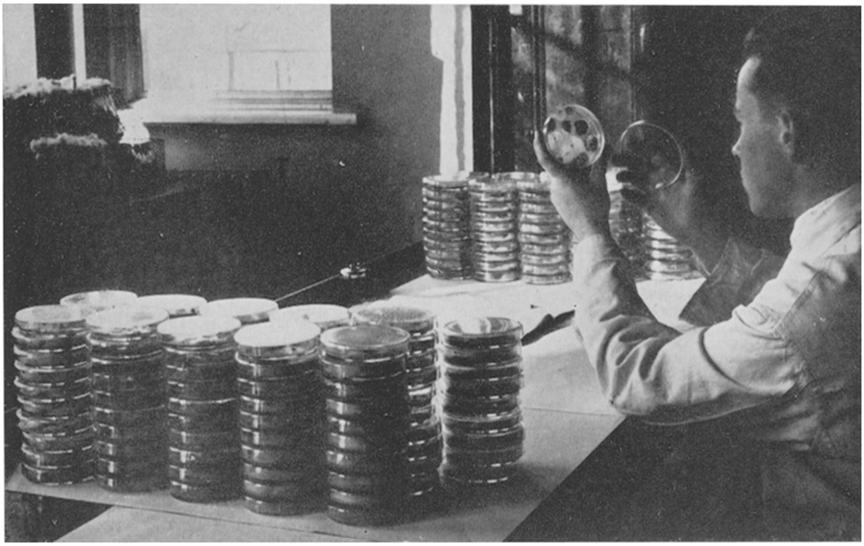

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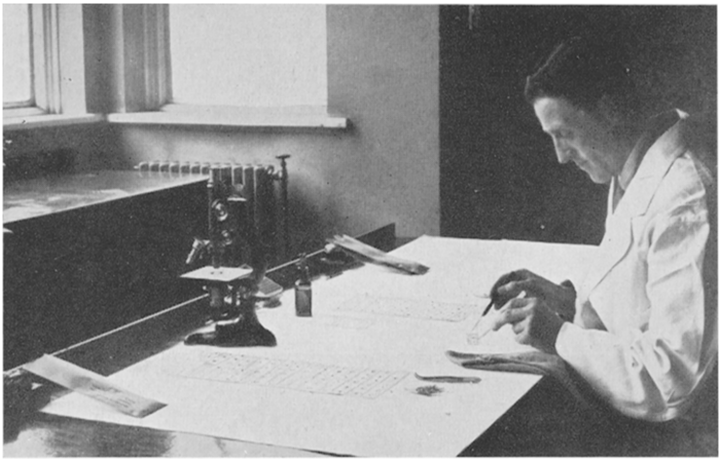

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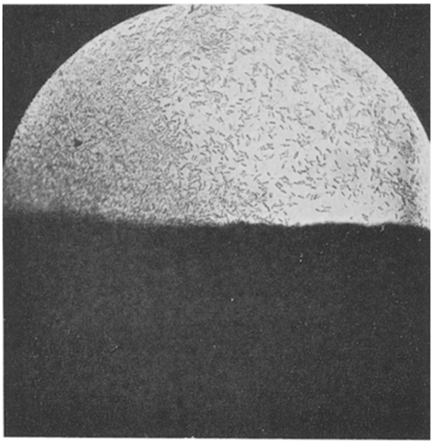

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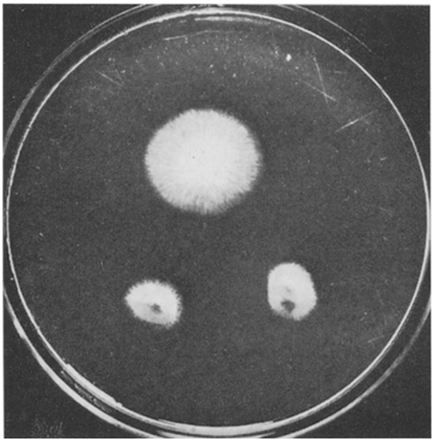

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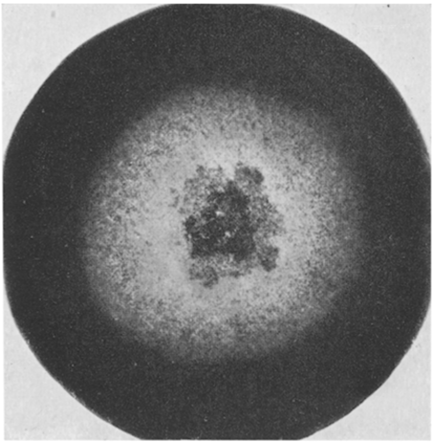

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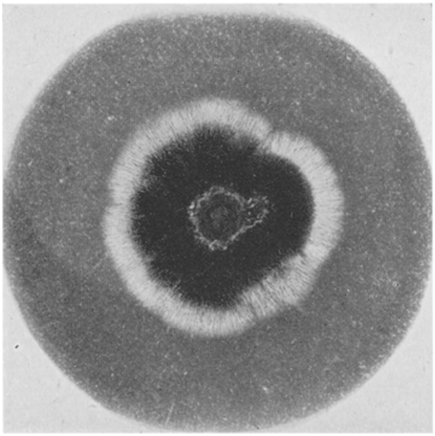

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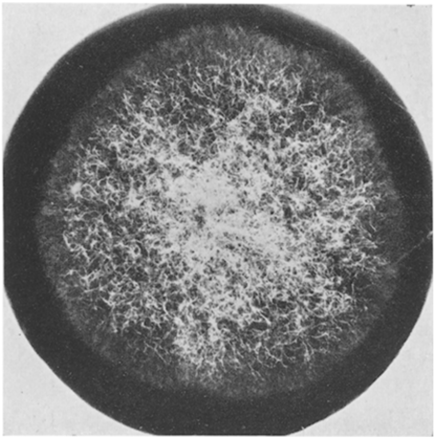

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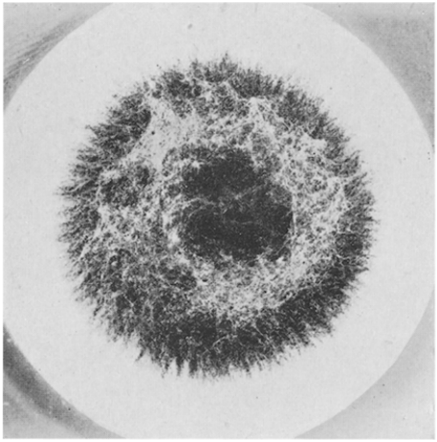

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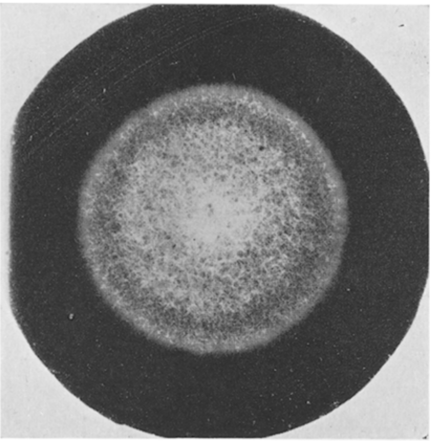

Fig. 13.

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EXPLANATION OF PLATES

Plate I

Fig. 1. The method employed for irradiating seed oats to encourage the production of spores of *Helminthosporium avenae*.

Fig. 2. The apparatus and laboratory set-up required for the examination of seed oats for contamination with *H. avenae*. The museum jar shown in the foreground is used for storing the Petri dishes in the incubator.

Fig. 3. Photomicrograph of the glume apices of an oat seed upon which *H. avenae* is growing and sporing freely.

Fig. 4. The apparatus and laboratory set-up required for the plating out of flax seeds upon malt extract agar in carrying out the Ulster method for flax seed examination.

Plate II

Fig. 5. The examination of the plates for the growth of seed-borne parasites of flax after an incubation period of five days.

Fig. 6. The apparatus and laboratory set-up required for the examination of ryegrass seed for contamination with *Phialea temulenta*.

Fig. 7. Macrospores of *P. temulenta* floating out from a contaminated seed into the surrounding water.

Fig. 8. Two typical cultures of *P. temulenta* (twelve days old) and a typical culture of *Pullularia pullulans* (five days old) are shown for purposes of comparison. *P. pullulans* is a common mould occurring on ryegrass seeds.

Plate III

Figs. 9–13. Typical cultures of the five seed-borne parasites of flax which can be identified by the Ulster method. Fig. 9, *Colletotrichum linicola*; Fig. 10, *Polyspora lini*; Fig. 11, *Botrytis cinerea*; Fig. 12, *Phoma* sp.; Fig. 13, *Fusarium lini*.